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**Evolutionary Divergence of Enzymatic Mechanisms for Tubulin Detyrosination**

*Graphical Abstract*

*Highlights*

- In contrast to VASH1, VASH2 does not require SVBP for detyrosinating activity
- Diversification of tubulin detyrosination coincides with the appearance of vertebrates
- TbVASH detyrosinates both \( \alpha \)- and \( \beta \)-tubulin tails of *Trypanosoma brucei*
- Parasites lacking TbVASH grow slowly and display severe morphological abnormalities

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*In Brief*

van der Laan et al. describe the evolutionary diversification of the VASH-related tubulin detyrosination mechanism. In addition, they show that this modification plays an important role in *Trypanosoma* parasites, where it regulates cell division and cell morphology.
Evolutionary Divergence of Enzymatic Mechanisms for Tubulin Detyrosination

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SUMMARY

The two related members of the vasohibin family, VASH1 and VASH2, encode human tubulin detyrosinases. Here we demonstrate that, in contrast to VASH1, which requires binding of small vasohibin binding protein (SVBP), VASH2 has autonomous tubulin detyrosinating activity. Moreover, we demonstrate that SVBP acts as a bona fide activator of both enzymes. Phylogenetic analysis of the vasohibin family revealed that regulatory diversification of VASH-mediated tubulin detyrosination coincided with early vertebrate evolution. Thus, as a model organism for functional analysis, we used Trypanosoma brucei (Tb), an evolutionarily early-branched eukaryote that possesses a single VASH and encodes a terminal tyrosine on both α- and β-tubulin tails, both subject to removal. Remarkably, although detyrosination levels are high in the flagellum, TbVASH knockout parasites did not present any noticeable flagellar abnormalities. In contrast, we observed reduced proliferation associated with profound morphological and mitotic defects, underscoring the importance of tubulin detyrosination in cell division.
and neurogenesis (Allaudi et al., 2017; Cambray-Deakin and Burgyne, 1987; Erck et al., 2009), where it accumulates mostly on stable MTs.

By using non-specific protease inhibitors, we found previously that tubulin detyrosinase is a cysteine protease. This type of protease is amenable to covalent inhibition by reactive chemical warheads, such as epoxide (Albeck and Kliper, 1997). Therefore, by fusing epoxide to different numbers of amino acids corresponding to the very C terminus of α-tubulin, we obtained compounds that inhibited detyrosinase activity in brain extracts. Using a biochemical approach consisting of covalent inhibitors compatible with click chemistry, we identified two members of the vasohibin family, VASH1 and VASH2, as tubulin detyrosinases (Allaudi et al., 2017). This was a major breakthrough in the field, opening up the possibility to perform direct functional studies and to finally get a better understanding of the evolutionary history of tubulin detyrosination.

Here we performed a phylogenetic analysis of the vasohibin protein family together with its chaperone, small vasohibin binding protein (SVBP). Our analysis revealed that regulatory diversification of VASH-mediated tubulin detyrosination coincided with early vertebrate evolution. In turn, we used Trypanosoma brucei (Tb), an evolutionarily early-branched eukaryote that possesses only a single VASH, as a model organism for functional analysis of tubulin detyrosination. We found that deletion of the VASH gene in these parasites results in severely defective morphogenesis and growth reduction. Although detyrosination in most species is specific to β-tubulin, trypanosomatid parasites are unique in that their β-tubulin also carries a tyrosine at the C-terminal end, which is also subject to removal (Schneider et al., 1997). We show that TbVASH removes the C-terminal tyrosine residue of both α- and β-tubulin, accommodating both tubulin tails in its enzymatic pocket. This specific trait provides the opportunity to design peptide-based inhibitors that preferentially target TbVASH. We propose that TbVASH may be an exploitable target for development of drugs against trypanosomatid-related diseases.

RESULTS

SVBP Is a Bona Fide Activator of VASH-Mediated Tubulin Detyrosination

To gain a better understanding of the evolutionary relationship between VASH enzymes and SVBP, we performed phylogenetic analysis of both families using sequences from diverse eukaryotic organisms. A protein phylogeny of the VASH family revealed three distinct clades: VASH1- and VASH2-related proteins from vertebrates and a clade grouping VASH sequences from invertebrates (Figure 1A). Of note, despite the documented presence of detyrosination in Caenorhabditis elegans and Drosophila melanogaster (O’Hagan et al., 2011; Warn et al., 1990), blastp results did not retrieve any VASH homologs, suggesting the existence of alternative tubulin detyrosinating enzymes. By using publicly available sequences, we also constructed a phylogenetic tree of SVBP (Figure 1B). We observed that the sequence homology within the vertebrate lineage was very high, and blastp search for SVBP sequences outside of vertebrates did not identify homologous genes. In view of the rounds of whole-genome duplication that occurred in early vertebrate evolution (Meyer and Schartl, 1999), the appearance of VASH1 and VASH2 proteins as separate genes may have emerged as a consequence of this phenomenon.

Because both VASH1 and VASH2 are enzymatically active (Figure S1A), and both enzymes bind to SVBP (Suzuki et al., 2010), we expressed a number of previously described natural isoforms of VASH2 (Sato, 2013) in HEK293 cells and performed pull-down assays using either glutathione S-transferase (GST) or GST-SVBP to more precisely map the interaction domains. We observed that full-length VASH2, N65-VASH2, and the C-terminally truncated VASH2-ΔC296 all associated with SVBP whereas ΔN104-VASH2 did not (Figure 1C). Furthermore, the association between SVBP and VASH was independent of its catalytic activity (Figure S1B). Because no evidence of similar VASH1 splice variants could be found in the Ensembl database (http://www.ensembl.org/), we generated homologous truncations of VASH1 based on the naturally occurring VASH2 isoforms. Similarly, full-length VASH1 and ΔN76-VASH1 interacted with SVBP (Figure 1D) whereas ΔN76–115-VASH1 did not. Similarly, to VASH2 the association of SVBP was independent of the enzymatic activity (Figure S1C). Altogether, these data indicate that binding occurs through the corresponding region of VASH2 (N-terminal residues 66–104) and VASH1 (N-terminal 76–115), a domain we termed the SVBP-binding site (SVBP-BS). The very recent structural data obtained for VASH1 and VASH2 (Adamopoulos et al., 2019; Liao et al., 2019) allowed structural alignment of the SVBP-BS region (Figure 1E) and confirmed the presence of important residues within this region that are involved in interaction with SVBP. Remarkably, co-transfection of either VASH1 or VASH2 with SVBP resulted in a marked increase in total tubulin detyrosination compared with cells transfected with the enzymes alone (Figures 2A and 2B). Similar activation was observed for the intermediate N-terminal truncations of VASH2, which still contained the SVBP binding site (Figure 2A). In contrast, activation of both VASH1s was completely lost upon removal of the

Figure 1. The Appearance of VASH1 and VASH2 Coincides with Branching of the Vertebrate Lineage

(A) Phylogenetic tree of the VASH protein family, constructed with homologous sequences of a number of model organisms.

(B) Phylogenetic tree of the SVBP protein family, constructed with homologous sequences of a number of model organisms.

(C) SVBP binds to the N-terminal domain of VASH2, comprising amino acids 65–104 (naturally occurring variants). Shown are GST-SVBP pull-down assays of ectopically expressed VASH2 constructs in HEK293 cells. Samples were immunoblotted using the indicated antibodies.

(D) SVBP binds to the N-terminal domain, comprising amino acids 76–115 of VASH1 (constructs that reflect the natural variants of VASH2). Shown are GST-SVBP pull-down assays of ectopically expressed VASH1 constructs in HEK293 cells. Samples were immunoblotted using the indicated antibodies.

(E) Structural alignment of VASH1 (PDB: 6J7B; red) and VASH2 (PDB: 6J4P; blue) using PyMOL software. The insets show one of the interaction domains with SVBP and the key residues involved in interaction.
N-terminal part that contains the SVBP binding site (Figures 2C and 2D), demonstrating that this part of the sequence is required for binding and mediates activation of the detyrosinating activity (Figures S2A and S2B).

**VASH2 Acts as an Autonomous Tubulin Detyrosinase**

Historically, most *in vitro* MT-based assays involved the use of tubulin purified from pig brain. However, brain tubulin carries high levels of detyrosination, greatly complicating analysis of VASH activity. In search of an unmodified substrate, we found that tubulin purified from *Spodoptera frugiperda*-derived Sf9 cells (Figure 3A) is fully tyrosinated; this modification could not be detected by mass spectrometry analysis (Figure 3B). Therefore, we generated an antibody against the detyrosinated C-terminal sequence of *Spodoptera frugiperda* α-tubulin (EGEGAEE) and tested its specificity compared with the commonly used anti-murine Δ1-tubulin antibody. Our results clearly show that detyrosinated Sf9 tubulin is specifically recognized by our newly developed antibody but not by the commonly used murine counterpart (Figure 3C). Therefore, using purified bacterially produced recombinant human VASH1 and VASH2 proteins and MTs assembled from purified Sf9 tubulin, we performed *in vitro* detyrosination assays. Although VASH2 displayed strong detyrosinase activity, recombinant hVASH1, surprisingly, appeared to be inactive (Figure 3D). Because we previously found that SVBP has the ability to increase detyrosination activity in human HEK2933 cells (Figures 2A and 2B), we co-transformed bacteria to produce recombinant VASH1 or VASH2 in complex with SVBP. Strikingly, we observed high detyrosinating activity for the VASH1/SVBP complex, whereas VASH1 alone was inactive (Figure 3E). In contrast to VASH1, VASH2 displayed autonomous detyrosinase activity that was further increased by ectopic addition of recombinant SVBP, supporting the notion that SVBP acts as a bona fide activator (Figures S3A and S3B, compare lanes 2 and 3). To test whether the activatory role of SVBP is mediated by increasing the affinity of VASH1 for MTs, we performed turbidimetry and co-sedimentation assays. Both approaches showed no measurable change in the affinity of VASH1 for MTs regardless of the presence or absence of SVBP (Figures S3C and S3D). Next we compared the kinetics of both enzymes in complex with SVBP. Unexpectedly, the SVBP/VASH1 complex displayed substantial detyrosinating activity toward the free tubulin dimer (Figure 3F, top panel; Figure S3E), whereas SVBP/VASH2 appeared to specifically modify microtubule filaments despite having much higher overall detyrosinating activity (Figure 3F, bottom panel; Figure S3F). Taken together, our data demonstrate that SVBP acts as an essential partner for VASH1-mediated tubulin detyrosinase whereas it is dispensable for VASH2.

**The Trypanosoma brucei VASH Homolog Catalyzes Tubulin Detyrosination**

Because the presence of two VASH genes and expression of a naturally detyrosinated α4-tubulin isoform in vertebrates complicates phenotypic analysis in human cells, we used *Trypanosoma brucei* as a model organism. Strikingly, although detyrosination in most species is specific to α-tubulin, trypanosomatid
parasites encode a β-tubulin, which also ends with a tyrosine and appears to be subject to removal (Schneider et al., 1997).

PTMs of tubulin, and specifically α-tubulin detyrosination, are well-documented in Tb, which assembles a number of elaborated MT-based structures, such as sub-cortical arrays, flagella, or mitotic spindles (Gull, 1999). We aligned the amino acid sequences of human VASH1 and VASH2 with that of TbVASH and found that the catalytic triad containing the essential cysteine residue was present within its central domain (Figure 4A). Overall, TbVASH displayed a slightly higher similarity to hVASH2 than to hVASH1 (Figure S4A) and, intriguingly, contained a putative nuclear localization signal (NLS) that was also present in HvASH2 (Figure 4B). In light of the closed mitosis in Trypanosoma, the presence of an NLS motif in TbVASH suggests that spindle detyrosination might occur directly in the nucleus. Expression of wild-type but not the catalytically dead variant of TbVASH in human HEK293 cells resulted in a strong increase in α-tubulin detyrosination, as observed by immunoblot analysis of protein lysates (Figure 4C) and by immunofluorescence analysis (Figure S4B). Using the in vitro assay described above (Figure 3), we showed that recombinant TbVASH completely removed the C-terminal tyrosine from α-tubulin and, similar to hVASH2, displayed autonomous catalytic activity (Figure 4D). Moreover, we found that TbVASH specifically detyrosinates microtubules and showed no significant activity toward free tubulin dimers (Figures 4E and S4C). Next, although no ortholog of the SVBP gene could be found in the T. brucei genome, we sought to test whether TbVASH contains the putative SVBP-BS. Based on the structural alignment of the N-terminal amino acids of TbVASH to the corresponding domains of vertebrate proteins, we found that previously described key residues were also present in TbVASH (Figure 4F). Pull-down assays using either full-length TbVASH or Tb-dN38-VASH and GST-tagged SVBP surprisingly demonstrated that
Figure 4. The VASH Gene in Trypanosoma brucei (Tb) Possesses Tubulin Detyrosinase Activity
(A) Sequence homology between TbVASH and human VASH1 and VASH2.
(B) Identification of the nuclear localization signal in TbVASH and human VASH1 and VASH2 sequences using an NLS mapper (nls-mapper.iab.keio.ac.jp).
(C) Detyrosinase activity of TbVASH. Ectopic expression of TbVASH and the enzymatically dead (Cys-Ala) protein in HEK293 cells. Twenty-four h after transfection, cells were collected and analyzed by immunoblotting with the indicated antibodies.

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the N-terminal domain contains the SVBP binding site. Deletion of the domain resulted in complete loss of interaction (Figure 4G). Strikingly, co-transfection of full-length TbVASH with SVBP in human HEK293 cells led to an increase in detyrosination activity, whereas Tb-ΔN38-VASH was insensitive to the presence of SVBP (Figure 4H). Thus, the domain required for interaction with SVBP appears to be already present in TbVASH despite the absence of a homologous gene for SVBP, which only appeared more than a billion years later.

Deletion of TbVASH Results in Elongated Parasites with Growth Defects

Using a recently developed CRISPR method (Beneke et al., 2017), we generated procyclic TbVASH-null mutant parasites, as demonstrated by PCR-based analysis (Figures S5A and S5B). To study the consequences of TbVASH deletion at the protein level, we raised Trypanosoma-specific antibodies for detyrosinated α- and β-tubulin (Figure 5A). As shown previously by immunoblot analysis, we observed that, in wild-type parasites, tyrosinated tubulin (YL1/2 staining) was strong in posterior ends and basal bodies as well as in growing new flagella (Sherwin et al., 1987). In contrast, detyrosination was observed globally over the entire cells, with the exception of the posterior ends (Figure 5B). Strikingly, staining for both detyrosinated α- and β-tubulin was lost in knockout (KO) parasites, suggesting that TbVASH catalyzes the modifications on both tubulin subunits (Figure 5B, bottom panel). Moreover, we observed increased labeling of tyrosinated tubulin, which became evenly distributed throughout the cells (Figure 5B). In agreement, immunoblot analysis of cell lysates further confirmed complete loss of α- and β-tubulin detyrosination in TbVASH KO cells and a simultaneous increase in the level of tyrosinated tubulin (Figure 5C). The activity of TbVASH toward β-tubulin was further validated using purified tubulin from Trypanosoma in an in vitro detyrosination assay (Figure S5C). In addition, TbVASH KO parasites did not display detectable changes in other tubulin modifications, such as acetylation or polyglutamylation (Figure 5C). Moreover, severe growth defects in KO parasites were observed and confirmed by proliferation assays (Figure 5D). Scanning electron microscopy further revealed abnormally elongated parasites in a Tb-VASH-deficient cell line (Figure 5E), reminiscent of a previously reported nozzle-like morphology (Hendriks et al., 2001). This phenotype was further confirmed by direct measurement of the distance between nuclei and posterior ends to assess parasite length (Figure 5F). Based on the previously described high levels of detyrosination in Trypanosoma flagella (Sasse and Gull, 1988), we anticipated functional defects in these structures. However, despite complete loss of detyrosination in KO parasites, the cells did not display major motility defects or striking flagellar abnormalities (Figure S5D). In addition, immunofluorescence analysis demonstrated that attachment of flagella to the cell body was not affected (Figure S5E). Taken together, although no obvious flagellar defects could be observed, we show that, in the absence of detyrosination, parasites displayed profound morphological defects and a strongly reduced proliferation rate.

TbVASH KO Parasites Display Mitotic Defects

To further study the mechanism underlying the growth defects, we analyzed the localization of TbVASH in parasites expressing inducible GFP-TbVASH. Remarkably, we found overexpressed GFP-TbVASH to be mostly localized in the nucleus (Figure 5A), which is consistent with the presence of a predicted NLS in its sequence (Figure 4B). Moreover, in parasites undergoing mitosis, we also detected GFP staining on the spindle, supporting the notion that spindle MTs are directly subjected to TbVASH-mediated detyrosination. This observation was corroborated by YL1/2 labeling of spindle MTs in KO parasites (Figure 6B), which was absent in the wild-type counterparts (Sasse and Gull, 1988). Moreover, spindle MTs were also positively labeled using antibodies specific to detyrosinated β-tubulin (Figure 5A). The bulk DNA content of TbVASH KO parasites revealed a marked broadening of G1 and G2 peaks (Figure S5B), suggesting cell cycle defects. Moreover, TbVASH KO cells displayed an increased forward scatter signal intensity consistent with increased cell size (Figure 6C). To confirm the potential link between morphological defects and cell cycle progression, we counted the number of nuclei and mitochondrial DNA, called kinetoplasts. We observed a decrease in post-mitotic (2 nuclei, 2 kinetoplasts [2N2K]) cells and appearance of anucleated parasites (0N1K), called zoids, in the TbVASH KO cell line (Figure 6D). We further analyzed chromosomal abnormalities using a fluorescence in situ hybridization (FISH) probe specifically targeting chromosome 1. TbVASH KO cells revealed a decrease in disomic cells and increased numbers of parasites having a single chromosome 1 (Figure 5E). Taken together, parasites lacking detyrosination displayed increased cell size, defective cell cycle progression, and abnormal chromosomal distribution. The striking increase in the number of zoids in the absence of TbVASH suggests completion of cell division without chromosome segregation. Given the absence of mitotic checkpoint mechanisms in Trypanosoma (Hammart, 2007), we further aimed to study the consequences of VASH2 depletion in human diploid cycling cells, such as RPE-1 cells. We optimized the experimental conditions for depletion of VASH2 in human cells...
Figure 5. Growth and Cell Cycle Progression Defects of TbVASH Knockout Parasites

(A) Characterization of the Trypanosoma brucei-specific detyrosinated tubulin antibodies. Shown is immunoblot analysis of HEK cells transfected with the indicated tubulin constructs. Twenty-four h after transfection, samples were collected and analyzed by immunoblotting with the indicated antibodies.

(B) Immunofluorescence analysis of wild-type (control) and TbVASH knockout (KO) parasites. Cells were stained for detyrosination on both α-tubulin (left panel) and β-tubulin (right panel).

(C) Immunoblot analysis of posttranslational modifications (PTMs) of microtubules in protein extracts obtained from parental and TbVASH KO parasites. α- and β-tubulin detyrosination was detected using specific antibodies.

(D) Culture growth curve of parental and TbVASH KO parasites over 4 days (n = 5; error bars represent SD). TbVASH KO parasites display severe growth defects.

(E) Scanning electron microscopy images of representative parental and TbVASH KO parasites. Scale bars, 10 μm.

(F) Cell size analysis of wild-type control and TbVASH KO parasites (n = 3; error bars represent SD).

(Figure S6C) and analyzed the effect of VASH2 knockdown on cell cycle distribution using a bromodeoxyuridine (BrdU) incorporation assay. In the absence of VASH2, we observed a marked increase in the number of BrdU-negative cells having 4n content, pointing to activation of a mitotic checkpoint (Figure S6D). Thus, we monitored the time in mitosis of each individual cell by time-lapse microscopy (Figure S6F). We found that, in the absence of VASH2, cells spent more time in mitosis (Figures S6E and S6F), suggesting that they have difficulties in progressing through mitosis. Finally, to evaluate potential compensatory mechanisms in these cells, we measured, by qPCR, the expression of relevant cell cycle- and cytoskeleton-related genes (Figure S6G). We found that CYC7 and CYC10 were upregulated, likely suggesting that these genes may contribute to the adaptation process (Liu et al., 2013).
Using the β-Tubulin Sequence as a Template for Developing TbVASH-Specific Inhibitors

Given the important functions of TbVASH in the life cycle of Trypanosoma, inhibition of tubulin detyrosination may provide a meaningful target for treatment of trypanosomatid-related diseases. Because TbVASH catalyzes the removal of the tyrosine not only from β- but also from α-tubulin (Figure 7A), this suggests that the enzymatic pocket differs significantly between human and Trypanosoma enzymes. Thus, we aimed to exploit this unique evolutionary feature to design specific peptide-based inhibitors of TbVASH. Given the presence of a highly reactive cysteine residue, VASHs are amenable to covalent inhibition by reactive chemical warheads such as epoxide (Eps). Using an in vitro detyrosination assay with recombinant TbVASH, we found that our previously described inhibitor of human VASHs, Eps-Y, also inhibits TbVASH (Figure S7A). By using the C-terminal end of Tb β-tubulin as a scaffold, we developed a series of compounds with higher affinity for TbVASH (Figures 7B and S7B). Surprisingly, changing the COOH group of tyrosine to NH₂ completely abolished the inhibitory effect of the compounds (Figure 7C, compare lanes 3 and 4), likely because of the loss of a stabilizing salt bridge between the inhibitor and the arginine surrounding the active pocket (Adamopoulos et al., 2019; Liao et al., 2019). Moreover, the tripeptide EQY alone or modified with a reactive NO₂ group (compound 3) did not result in any detectable TbVASH inhibition (Figure 7C, compare lanes 3, 5, and 6).

Finally, Q-Eps-Y did inhibit Tb-VASH with an estimated IC₅₀ around 50 μM (Figure 7D). To assess Q-Eps-Y on human VASHs, we used the human CHLQ15 cell line, in which detyrosination mostly depends on hVASH1 and hVASH2 enzymes (Nieuwenhuis et al., 2017). Although Eps-Y inhibited detyrosination at 25 μM, the glutamine-containing compound (compound 4) at the same concentration did not affect the level of detyrosination (Figure 7E). To rule out the possibility that the reduced effect of compound 4 in this system may be due to decreased cell penetration, we overexpressed TbVASH in human HEK293 cells and compared the inhibition of both compounds. As expected, both inhibitors resulted in similar inhibition of TbVASH-dependent detyrosination (Figure 7F). Finally, based on recently published structural data of hVASH1 and hVASH2 bound to SVBP (Liao et al., 2019), we modeled Tb-VASH using both structures as a template (Figure S7C). Because our data provide evidence that hVASH2 is more closely related to TbVASH, we further present the model of the Eps-Y ligand in the catalytic...
pocket of Tb-VASH based on the structure of VASH2 (Figure 7G).
Thus, the unique β-tubulin sequence of TbVASH may be a promising scaffold for rational drug design to limit multiplication of the parasite. Such compounds could be further developed and assessed as innovative drugs to treat trypanosomid-related diseases and should have improved safety profiles because of their selectivity toward parasites enzymes.

**DISCUSSION**

Here we found that VASH1 requires binding of SVBP tubulin detyrosinating activity, whereas VASH2 has autonomous activity. Moreover, we demonstrate that SVBP acts as bona fide activator of both enzymes. Because the presence of two VASH genes in vertebrate species complicates phenotypic analysis, we used Trypanosoma brucei, a divergent eukaryote that possesses only a single VASH gene, as a model organism. We show that TbVASH knockout parasites display severe growth abnormalities likely related to mitotic progression defects. Moreover, we present a rational method and the proof of concept for design of inhibitors of TbVASH based on a unique evolutionary feature of β-tubulin in Trypanosoma that could potentially serve further drug development.

Vertebrate genomes show evidence of widespread gene duplication (Panopoulou et al., 2003), which might explain the appearance of two independent VASH genes specifically in vertebrates. Typically, in such cases, one copy either degenerates and becomes inactive or undergoes functional specialization, allowing development of more complex regulatory mechanisms (Dehal and Boore, 2005). Because both VASH1 and VASH2 are enzymatically active (Figure S1A), these observations may reflect the increased complexity of the regulatory mechanisms governing α-tubulin detyrosination. Moreover, the appearance of SVBP in vertebrates may have further contributed to the

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Figure 7. Using Unique Features of T. brucei to Develop Peptide-Based Inhibitors of TbVASH
(A) Sequence alignment of the C-terminal tails of both human α Tub1A and TubB2A genes with, respectively, α- and β-tubulin from T. brucei α-tubulin show large difference in β-tubulin tail sequence.
(B) Schematic drawing of Q-Eps-Y (compound 4). The Q-Eps-Y compound was designed based on the C-terminal sequence of T. brucei β-tubulin.
(C) In vitro detyrosination assay using recombinant TbVASH in the absence or presence of 100 μM compound. The assay was performed in the presence of 0.5 μM microtubules. Reactions were stopped after 15 min and analyzed by immunoblotting with the indicated antibodies.
(D) Dose-response analysis of compound 4 as an inhibitor of TbVASH. Shown is an in vitro detyrosination assay using recombinant TbVASH in the presence of increasing concentrations (2–100 μM) of compound 4. The assay was performed in the presence of 0.5 μM microtubules. The reactions were stopped after 15 min and analyzed by immunoblotting with the indicated antibodies.
(E) Dose-response analysis of compounds 1, 3, and 4 in cellulo. HEK293 cells were treated with increasing amounts of inhibitors and collected 24 h after treatment. The cells were collected and analyzed by immunoblotting with the indicated antibodies.
(F) Inhibition of ectopically expressed TbVASH in cells. Shown is ectopic expression of TbVASH in HEK293 cells and 24 h treatment with the indicated inhibitor. Following treatment, cells were collected and analyzed by immunoblotting with the indicated antibodies.
(G) Structural model of TbVASH with the catalytic domain bound to compound 1 (Eps-Y). Human VASH2 was used as a template to model TbVASH because both enzymes seem to be functionally more related.
increased complexity of neuronal organization (Aillaud et al., 2017; Erck et al., 2005; Gundersen and Bulinski, 1986b; Iqbal et al., 2019). In contrast, HVAS2, which acts as an autonomous detyrosinase, seem to be more related to TbVASH and does not depend on the presence of SVBP for activity. In view of the detyrosinase, we show that a functional binding site is already present in TbVASH and that the interaction leads to increased activity. Previ-
ous study have suggested that the mitotic spindle in Trypano-
soma is composed exclusively of detyrosinated microtubules. This observation was based on the absence of YL1/2 labeling, which specifically recognizes tyrosinated a-tubulin (Sasse and Gull, 1988). The authors proposed that the spindle is either assembled from already modified tubulin or, alternatively, undergoes rapid post-assembly detyrosination. Our findings that TbVASH does not modify tubulin dimers and that it shows strong localization to the nucleus indicate that spindle detyrosination takes place after MT assembly. Interestingly, despite the absence of a SVBP gene in the Trypanosoma brucei genome, we show that a functional binding site is already present in TbVASH and that the interaction leads to increased activity. This suggests that, during evolution, the appearance of the SVBP gene was sufficient to further increase the regulatory complexity of the tubulin detyrosination mechanism, underscor-
ing the functional importance of this modification.

Unexpectedly, TbVASH mutant parasites did not display any visible dysfunction of their flagellum, either in motility or in structure (Figure 5). However, the mutants had abnormally long post-
terior regions. Increased MT polymerization has been proposed as a potential mechanism for trypanosome cell elongation (Sher-win and Gull, 1989a, 1989b). Because, in the current study, we deleted the enzyme involved in detyrosination, the observed in-
crease in YL1/2 labeling over the entire cell cannot be interpreted as a marker of newly assembled MTs. However, with respect to the thinned shaped of the elongated parasites, we speculate that this results from sliding of MTs past each other, caused by dys-
regulation of VASH-mediated tubulin detyrosination. This opens the possibility that the differential status of detyrosination regu-
lates motor proteins that cross-link and slide microtubules. These morphological abnormalities might also be a conse-
quence of altered cell cycle progression, as demonstrated previously with mutant parasites displaying a “nozzle-like” morphology (Hammarton et al., 2004; Liu et al., 2013; Tu and Wang, 2004). Furthermore, our data demonstrate that TbVASH mediates detyrosination not only of a but also of b-tubulin and, therefore, controls the parasite’s cell cycle and mitotic divi-
sion. This makes detyrosination an important signal for parasites to undergo division, likely through pleomorphic functional activities, which include modification of mitotic spindle MTs (Figure 6).

A logical path for developing new therapeutic approaches is to focus on specific features that distinguish the parasites from their human host. Although detyrosination in most species is specific to a-tubulin, both a- and b-tubulins of trypanosomatid parasites are subjected to detyrosination catalyzed by TbVASH (Figure 5). Moreover, the b-tubulin sequence differs significantly from human and Trypanosoma a-tubulin sequences, suggesting that the parasite’s enzyme has a distinct pocket for catalysis. Because of the presence of a highly reactive cysteine residue in the cleft, this type of protease is amenable to covalent inhibi-
tion by reactive chemical warheads such as Eps (Albeck and Kliper, 1997). In Trypanosoma, b-tubulin ends with -EQY, in which the negatively charged penultimate glutamate residue is replaced by the non-charged glutamine. Therefore, we gener-
at ed Q-Eps-Y, which closely resembles the C-terminal sequence of Tb b-tubulin, and show that it preferentially inhibits TbVASH.

Further chemical optimization of our compound could provide a potent peptide-based inhibitor of Trypanosoma with improved toxicity profiles compared with currently available therapies. Such an approach may be extended to other parasites, such as Leishmania, that also possess b-tubulin with a very C-terminal tyrosine residue likely subject to detyrosination.

In summary, we found that SVBP acts as a bona fide activator of VASH-mediated tubulin detyrosination, and this is yet another example of the regulatory mechanisms that control the activity of tubulin-modifying enzymes, as we showed recently for glutamy-
lases (Bompard et al., 2018). We show that hVASH2 acts as an autonomous detyrosinase and that, in many respects, it has characteristics similar to TbVASH protein. Although the role of detyrosination in coordinating cell morphogenesis and cell divi-
sion has already been proposed, our data regarding TbVASH, which modifies cortical and spindle MTs, provides a molecular link between the two processes. Moreover, using an evolutionary unique feature of the Tb b-tubulin C-terminal tail, we provide a method for rational design of small-molecule inhibitors as drugs against trypanosomatid-related diseases.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **LEAD CONTACT AND MATERIALS AVAILABILITY**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Cell culture
  - Trypanosoma cell culture and generation of transgenic lines
- **METHOD DETAILS**
  - Transfection of human cells
  - Multiple alignments and phylogenetic analysis
  - Protein expression and purification
  - In vitro characterization of detyrosination activities
  - Flow cytometry
  - SF insect cells culture and protein production
  - Transmission electron microscopy
  - Fluorescence in situ hybridization (FISH)
  - Quantitative PCR
  - Immunofluorescence labeling
  - Mass spectrometry
  - Trypanosoma cell culture and generation of transgenic lines
  - Microscopy
  - Reagents and antibodies
  - Plasmids
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **DATA AND CODE AVAILABILITY**
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.11.074.

ACKNOWLEDGMENTS

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The work was funded by the following expression vectors; and J. Gaertig (University of Georgia) for critical reading.

AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

A patent called “In vitro screening assay of tubulin detyrosinase inhibitors” that further describes the use of the assay has been filed.

REFERENCES


### KEY RESOURCES TABLE

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(Continued on next page)
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Krzysztof Rogowski (krzysztof.rogowski@igh.cnrs.fr). All unique/stable reagents will be made available on request but we may require a payment and/or a completed Materials Transfer Agreement if there is potential for commercial application.

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**Software and Algorithms**

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Trypanosoma cell culture and generation of transgenic lines

Q24 Procyclic T. brucei brucei 29-13 (Wirtz et al., 1999) and SmOx P9 pTB011 (Geneke et al., 2017) cell lines were cultured at 27°C in SDM 79 medium (Brun and Schonenberger, 1979) supplemented with 10% decomplemented fetal bovine serum (FBS) and 7 μg/ml hemin. To generate TbVASH null mutants, PCR reactions for sgRNA templates and donor DNAs were used according to the published toolkit for genome editing in kinetoplastids (Geneke et al., 2017). Primers were designed using online tools (LeishGEdit.net and http://grna.ctedg.uga.edu).

METHOD DETAILS

Transfection of human cells

Plasmid and siRNA transfections were performed with JetPEI (Polyplus) and Interferin (Polyplus) respectively, according to manufacturer’s guidelines. Cells were collected 48 hours after transfection for immunoblot and immunofluorescence analysis.

Multiple alignments and phylogenetic analysis

Multiple sequence alignments of the VASH and SVBP protein families were performed using Unipro UGENE suite with MUSCLE using default parameters. An alignment of human and T. brucei/VASH proteins was produced using Clustal Omega with default parameters, 200 rounds of iteration, and followed by quality assessment. In all cases, alignments were manually refined and figures were generated using the ESPript 3.0 server (http://espript.ibcp.fr). Manually refined multiple sequence alignments of protein sequences were used for molecular phylogeny analyses using IQ-TREE (http://iqtree.cibiv.univie.ac.at), using maximum likelihood (Trifinopoulos et al., 2016). Branch support was tested with bootstrap analysis (100 replications), and by two single branch tests, i.e., SH-like approximate likelihood ratio test (10) and a Bayes test.

Protein expression and purification

Human hVASH1 and hVASH2 coding regions were cloned in pCold vector with a polyhistidine tag. The SVBP coding region was also cloned both into pGEX vector for GST-tag protein and into modified pET to produce untagged variant. BL21 bacteria were transformed and induced with IPTG for either overnight at 18°C (pCold vectors) or for 4 hours at 30°C (pGEX or pET vectors). Bacteria were collected and disrupted using a HTU-DIGI-F press (Heinemann). Recombinant proteins were purified using nickel-based affinity chromatography (IMAC) according to the manufacturer’s protocol (GE Healthcare).

In vitro characterization of detyrosination activities

Q27 St9 cells were grown, lysed and used for tubulin purification by affinity chromatography as previously described (Widlund et al., 2012). Microtubules were obtained with taxol and stored at ~80°C until further use. In vitro analysis of detyrosination activities was performed using recombinant hVASH1, hVASH2 and TbVASH proteins. Detyrosination assays were performed in 50 mM sodium phosphate buffer in presence of 0.5 μM microtubules obtained from St9 tubulin. Reactions were stopped by addition of the denaturing loading buffer followed by 5 min incubation at 95°C and loaded on SDS-PAGE for immunoblot analysis.

Flow cytometry

For flow cytometry analysis of parasites, single cells were suspended at a density of 10^6 ml^-1 in 70% methanol, 30% PBS and were incubated at 4°C overnight. Cells were washed in 10 ml of cold PBS, resuspended in 1 ml of PBS containing 10 μg ml^-1 propidium iodide and 10 μg ml^-1 RNase A (Sigma), and incubated at 37°C for 45 min. Bulk DNA analysis by flow cytometry was performed with a Mitenyi MACS quant and analysis performed with FlowJo software.

Sf insect cells culture and protein production

St9 and Sf21 cells were grown in EX-CELL® 420 Serum-free medium (14420C, Sigma-Aldrich). Cells were maintained between 1 and 10 million cells/ml in shake flasks, at 28 degrees and 140 rpm agitation. For production of human hVASH1, hVASH2 and SVBP cDNAs were cloned into pFastBac vector MAX efficiency DH10Bac. For infection, 40 million Sf21 cells (in 20 ml) were infected with combinations of P2 encoding hVASH1, hVASH2 and SVBP. Coexpression of hVASH1 and hVASH2 with SVBP was performed by mixing 1:1 with respective P2 supernatants. After 48 hours, infected cells were collected by centrifugation and analyzed by immunoblotting.

Transmission electron microscopy

For ultrastructural analysis, parasites were fixed in 3.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.4) overnight at 4°C. The samples were rinsed in phosphate buffer and post-fixed in 1% osmic and 0.8% potassium ferrocyanide for 2 hr in the dark at room temperature (RT). After rinsing in phosphate buffer, samples were dehydrated in a series of ethanol solutions (30%-100%) and embedded in EmBed-812/DER 736. Sections were cut on a Leica-Reichert Ultracut E microtome and counterstained with uranyl acetate and lead citrate. Images were acquired with a Hitachi 7100 transmission electron microscope at MRI-COMET Montpellier (France).
Fluorescence in situ hybridization (FISH)

Control and knockout parasites were fixed in 4% paraformaldehyde and dehydrated in serial ethanol baths (50%–100%). The probe targeting s/[l]-tubulin gene cluster on chromosome 1 (Bessat and Ensfeld, 2009) was labeled with tetramethyl-rhodamine-5-dUTP (Roche®) by using the Nick Translation Mix (Roche®). Slides were then hybridized with a heat-denatured DNA probe under a sealed rubber frame at 94°C for 2 min and then overnight at 37°C. The hybridization solution contained 50% formamide, 10% dextran sulfate, 2X SSPE, 250 mg/ml salmon sperm DNA and 100ng of labeled double strand DNA probe. After hybridization, cells were sequentially washed in 50% formamide-2 X SSC at 37°C for 30 min, 2X SSC at 50°C for 10 min, 2X SSC at 80°C for 10 min, 4X SSC at room temperature. Slides were finally mounted in Vectashield with DAPI. To analyze copy number of chromosome, three independent experiments were performed and ~150 cells were counted in each replicate.

Quantitative PCR

Total RNA was isolated with TRIzol reagent (Invitrogen). Reverse transcription was carried out with random hexanucleotides (Sigma) using PrimeScript RT reagent Kit (Takara). Quantitative PCRs were performed using LightCycler SYBR Green I Master mix (Roche) on LightCycler apparatus (Roche). All primers used were intronspansing (primer sequences in Supplemental Information). The relative amount of target cDNA was obtained by normalizing using geometric averaging of multiple internal control genes (ACTB, HPRT, HMBS, GAPDH, and SDHA). For mRNA expression analysis of Trypanosoma Brucei all qPCR data were obtained by subtraction of genomic amplification.

Immunofluorescence labeling

Methanol fixed U2OS cells were analyzed using a Zeiss AxioImager Apotome microscope after standard immunofluorescence overnight staining. Parasites were fixed for 20 min with 4% (w/v) paraformaldehyde in PBS and allowed to settle onto teflon slides (Thermo Fisher Scientific) before permeabilization for 10 min with 0.2% Triton X-100 in PBS, and blockage with 2% (v/v) FBS in PBS. Cells were incubated overnight with primary antibodies. Cells were washed three times in PBS and incubated 1h with Alexa Fluor 488-conjugated goat anti-rat, goat anti-mouse and Alexa Fluor 555-conjugated goat anti-rabbit secondary antibodies (Invitrogen) and then washed three times in PBS, stained with DAPI and mounted.

Mass spectrometry

Proteins were separated on SDS-PAGE gels (10% polyacrylamide; Mini-Protean TGX Precast Gels; Bio-Rad) and stained with Page Blue Stain (Fermentas). Gel lanes were cut into several gel pieces and destained by three washes in 50% acetonitrile and 50 mM triethylammonium bicarbonate. Proteins were in-gel digested using trypsin (0.5 μg/band; Gold; Promega).

Peptides were then analyzed online by nano-flow HPLC-nanoelectrospray ionization using a Qexactive Plus mass spectrometer (Thermo Fisher Scientific) coupled to a nano- LC system (U3000-RSLC, Thermo Fisher Scientific). Desalting and preconcentration of samples were performed on-line on a Pepmap® precolumn (0.3 x 10 mm; Dionex). A gradient consisting of 0%–40% B in A for 100 min (A: 0.1% formic acid, 2% acetonitrile in water, and B: 0.1% formic acid in 80% acetonitrile) at 300 nl/min, was used to elute peptides from the capillary reverse-phase column (0.075 x 150 mm, Pepmap®, Dionex). Data were acquired using the Xcalibur software (version 4.0). A cycle of one full-scan mass spectrum (375–1,500 m/z) at a resolution of 70,000 (at 200 m/z), followed by 12 data-dependent MS/MS spectra (at a resolution of 17,500, isolation window 1.2 m/z) was repeated continuously throughout the run. Raw data analysis was performed using the MaxQuant software (version 1.5.5.1) with standard settings. Used database consist of Spodoptera frugiperda entries from Uniprot, G3CKA7 sequence (Uniprot) deleted from 1 to 6 amino acids in

Trypanosoma cell culture and generation of transgenic lines

For transfection, log phase cultures were washed and resuspended in 500μL of ZPSM buffer pH 7 (132 mM NaCl, 8 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 0.5 mM Mg(CH3COO)2, 90 μM C4H6CaO4) prior to electroporation (exponential protocol, 25μF, 1.5 kV, with a Bio-Rad gene pulser 2 electroporator) in 0.4 cm gap cuvettes with generated DNA constructs. To generate TbVASH RNAi constructs was induced with 1 μg/ml doxycycline. Amplifications of 5’ and 3’ sgRNA templates were performed with primers ML40-ML42 and ML41-ML42, respectively, and repair DNAs with primers ML43-ML44 using pPOTv7-phleomycin and pPOTv7-hygromycin templates (Dean et al., 2015). A total of four PCR products were purified using Wizard SV Gel and PCR clean up system (Promega) and heat-sterilized before transfection of 35 μg of DNA into 3.107 SmOx P9 pTB011 procyclic cells. After parasites selection with 2.5 μg/ml phleomycin and 50 μg/ml hygromycin, genomic DNA was extracted to monitor the loss of TbVASH locus and integration of the resistance cassettes.

Please cite this article in press as: van der Laan et al., Evolutionary Divergence of Enzymatic Mechanisms for Tubulin Detyrosination, Cell Reports (2019), https://doi.org/10.1016/j.celrep.2019.11.074
Microscopy
All microscopy was performed on Montpellier RIO imaging platform and images processed using OMERO. Scanning electron microscopy (S4000 Hitachi) of T. brucel parasites was performed on freshly prepared parasites. Confocal imaging was performed on a Zeiss LSM780 confocal microscope on paraformaldehyde-fixed T. brucel samples.

Reagents and antibodies
TvBASH inhibitors synthesis
All of the Fluorenylmethyloxycarbonyl (Fmoc) protected amino acids and (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazol-4,5-b) pyridinium3-oxid hexafluorophosphate (HATU) were provided by Iris Biotech GmbH. Piperidine, N.N-Dioxide propylthelyamine (DIEA), TFA, Trisopropylsilane (TIS), dichloromethane (DCM), 1,2-Dichloroethane (DCE) and N,N-Dimethylformamide (DMF) N-methylpyrolidinone (NMP) and ethyl ether (EtOEt) were provided by Sigma Aldrich. AmphiSpheres 40 RAM 0.38 mmol/g was purchased from Agilent Technologies. Solvents used for HPLC and LC/MS were of HPLC grade. All final compounds were purified by reversed-phase 18 HPLC and the purity assessed by analytical reversed-phase HPLC.

SPPS procedure
Peptide synthesis was performed using a standard SPPS protocol (Amblard et al., 2006). Each synthesis was performed using FmocRink amide AmphiSpheres 40 resin (0.3(mmol/g). The Fmoc protected amino acids (4 eq), HATU (4 eq) and DIEA (6 eq) were added to the syringe reactor and the mixture was stirred for 1 hour at room temperature. After each coupling reaction, the peptide-resin was submitted to two 5 min deprotection cycles with DMF/piperidine 80/20 v/v solution.

Compounds purification
All crude compounds were purified by preparative HPLC (Waters 4000 apparatus) on a C18 reversed-phase column (C18 Deltapak 100 mm x 40 mm, 15 µm). The Fmoc protected amino acids (4 eq), HATU (4 eq) and DIEA (6 eq) were added to the syringe reactor and the mixture was stirred at room temperature overnight. The RM was then poured in the 100ml of a 1/1/0.001 V/V/V mixture of ACN/H2O/TFA and was then poured in 50 mL of EtOAc. The organic phase was then washed with a mixture of water/brine 50/50 and 2 times with saturated solution of NaHCO3. The organic phase was then washed with brine solution, dried of MgSO4 and concentrated dry to afford 182 mg of Compound 1 (0.48mmol) were dissolved in 20ml of TFA and stirred at RT for 1h. The TFA was then concentrated under reduced pressure and the obtained oil was purified by semi-preparative HPLC with a gradient to afford after lyophilisation 70 mg of 1 compound as white powder. The obtained crude product was used for the next reaction without purification.

Synthesis of HO-Epo-Tyr-OtBu (1’)
948mg (4mmol) of HTyrOtBu were dissolved in 10 mL of DMF followed by 0.5ml of DIEA. In a separate beaker were dissolved 132mg (4mmol) of (x) trans-Epoxysuccinic acid (HO-Epo-OH) in 1ml of DMF followed by 1.5ml of DIEA and 1.520 gr (4mmol) of HATU. The mixture was stirred at room temperature overnight. The mixture was added in a drop-wise manner to the HTyrOtBu solution. The reaction mixture was then stirred for additional 2 hours overnight. The RM was diluted with 10ml of a 1/1/0.001 V/V/V mixture of ACN/H2O/TFA and was then poured in 50 mL of EtOAc. The organic phase was then washed with a mixture of water/brine 50/50 and 2 times with saturated solution of NaHCO3. The organic phase was then washed with brine solution, dried of MgSO4 and concentrated dry to afford 182 mg of the product as white powder. The obtained crude product was used for the next reaction without purification.

Synthesis of Ac-Glu-Gln-Tyr(NO2)-OH (3)
Fmoc-Tyr(NO2)-OH was attached to 150mg 2-Chlorotrityl chloride resin (cas42074-68-0) 100-200 mesh charged at 1.6 mmol Cl−/g resin by dissolving 0.7 eq. of Fmoc-Tyr(NO2)-OH and 2 eq. of DIEA in DMF and mixing in with the resin in a syringe reactor for 2 hours.

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The resin was then washed 3 times DMF, 3 times DCM and SPPS procedure was performed to obtain intermediate 3’ on the resin. The intermediate amine function was acetylated by a DCM/Ac2O 50/50 v/v treatment and treated with a Pip/DMF 20/80 v/v solution to remove eventual adducts to the unprotected OH function of the nitro-tyrosine. The compound was the cleaved from the resin by a 100ml TFA/DCM 50/50 v/v treatment for 2h. The solution was evaporated and 50 mL of EtOEt were added to the remaining oil. Compound 3 precipitated and was removed by centrifugation and then purified by preparative HPLC to obtain 11 mg as slightly green powder.

**Synthesis H2N-Gln-Epo-Tyr-OH (4)**

Fmoc-Gln(Trt)-OH was attached to 400 mg of rink amide resin (0.38 mmol/gr) according to SPPS and the Fmoc group was deprotected by DMF/pip 80/20 v/v treatment. Then 105mg of compound 1” (0.3 mmol) were dissolved in 5ml of DMF followed by 150ul of DIEA and 144mg of HATU. The mixture was stirred at RT for 2 min and was then added to the resin in a dropwise manner and stirred for 4 hours. After standard washing steps the peptide was cleaved from the resin by treatment with TFA/DCM 1/1 v/v solution for 2 hours. The solution was concentrated under reduced pressure. 50 mL of EtOEt were added to the remaining oil and the precipitated crude compound 4 was isolated by centrifugation and then purified by preparative HPLC to afford 9.5mg of the product as white crystals.

**Plasmids**

All DNA sequences were retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/) and TriTryp databases (https://tritrypdb.org/tritrypdb/). The following sequences were cloned from the human and Trypanosoma brucei cDNA and inserted into pCold, EGFP or HA tagged vectors. All DNA constructs were cloned by PCR and inserted in pRK5-HA or pEGFP-C1 for expression in HEK293 and U2OS cells. For bacterial production of recombinant proteins, open reading frames were cloned into pCold vector. Point mutations were introduced into hVASH1 and hVASH2 to generate enzymatically dead versions of the proteins (Cysteine to Alanine) by a PCR-based quick-change method.

**Quantification and Statistical Analysis**

Where relevant, data is represented as the mean of the indicated number of independent replicates. Statistical differences were assessed with an multiple Student’s t test (two tailed). Calculations were performed using Prism 7, version 7.0c (GraphPad). Data are represented as means ± standard errors of the mean (SEM) or standard deviations (SD) otherwise mentioned. n indicated the number of individual experiments. P values are indicated within figure legends and on figures as followed: * < 0.05, ** < 0.01, *** < 0.001. Numbers of counted cells are indicated within figure legends when required.

**Data and Code Availability**

The published article includes all datasets generated or analyzed during this study.